Plasma constituents and mortality in rat pups given chronic insulin via injection, pellet, or osmotic minipump

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Abstract: Two studies compared the glucose responses of 9-day-old rats given subcutaneous insulin, either continuously or via daily injection, for 10 days. In Experiment 1, implanted pellets released a total of 0, 1.9, or 5.7 U insulin/kg the first 24 h. Injected doses were larger, 0 or 8 U/kg. Injections caused no deaths, but insulin-releasing pellets caused high mortality within 24 h. Pups surviving the pellets were normoglycemic by treatment day 8. In Experiment 2, pups received 0.184 U of insulin daily, approximately 8 U/kg at 9 days, via either injection or osmotic minipump. All pups survived. Injected pups were hypoglycemic 2 h postinjection through treatment day 10, whereas pups with insulin minipumps were normoglycemic by day 5. Insulin injections, but not minipumps, lowered plasma triglycerides on day 10. To examine age differences in response to insulin, additional pups and adults received daily injections of 0 or 8 U/kg for 10 days. All survived. Insulin lowered plasma glucose more in pups than in adults and reduced triglycerides in pups but not in adults. The rapid development of normoglycemia in pups with insulin minipumps, compared with pups injected daily with the same dose, suggests that continuous early insulin may produce insulin resistance.

Key words: route of insulin administration, insulin resistance, mortality, plasma glucose, development.

Résumé : Deux études ont comparé les réponses au glucose de rats âgés de 9 jours auxquels on a administré de l'insuline sous-cutanée soit en continu, soit sous forme d'injection quotidienne pendant 10 jours. Dans l'expérience 1, les pellets implantés ont libéré un total de 0, 1,9 ou 5,7 U d'insuline/kg pendant les premières 24 heures. Les doses injectées ont été plus fortes, 0 ou 8 U/kg. Les injections n'ont pas causé de décès, mais les pellets ont entraîné un haut taux de mortalité en deçà de 24 heures. Les ratons ayant survécu ont été normoglycémiques au plus tard le jour 8 du traitement. Dans l'expérience 2, les ratons ont reçu 0,184 U d'insuline quotidiennement, approximativement 8 U/kg au jour 9, soit par injection, soit par une mini-pompe osmotique. Tous les ratons ont survécu. Les ratons injectés ont été hypoglycémiques deux heures après l'injection jusqu'au jour 10 du traitement, tandis que les ratons ayant reçu l'insuline par le biais des mini-pompes ont été normoglycémiques au plus tard le jour 5. Les injections d'insuline, mais pas les mini-pompes, ont diminué les taux de triglycérides plasmatiques le jour 10. Pour examiner les différences selon l'âge de la réponse à l'insuline, d'autres ratons et des adultes ont reçu des injections quotidiennes de 0 ou 8 U/kg pendant 10 jours. Tous ont survécu. L'insuline a diminué les taux de glucose plasmatique davantage chez les ratons que chez les adultes, et réduit les taux de triglycérides chez les ratons uniquement. Le développement rapide de la normoglycémie chez les ratons portant les mini-pompes à insuline, comparativement aux ratons injectés quotidiennement avec la même dose d'insuline, donne à penser que l'administration précoce continue d'insuline pourrait provoquer une insulinorésistance.

Mots clés : voie d'administration de l'insuline, insulinorésistance, mortalité, glucose plasmatique, développement.

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Introduction

Insulin is an anabolic hormone that plays a significant role in the metabolism of carbohydrates, proteins, and lipids. One activity is to facilitate the entry of glucose into adipose, muscle, and other tissue, thus helping to regulate plasma glucose levels. It is well known that a chronic insulin deficit results in elevated plasma glucose levels and other symptoms of diabetes, whereas excessive insulin activity may lower plasma glucose to a point where the brain is starved for energy and a life-threatening insulin shock syndrome occurs.

Several investigators have examined the ontogeny of insulin's appearance and actions. Insulin appears to facilitate the

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development of brain tissue by aiding the processes of brain myelination and glial differentiation (Sena and Ferret-Sena 1995), and fetal concentrations of circulating brain insulin are higher than neonatal or adult values in rats, mice, and rabbits (Bernstein et al. 1984; Devaskar et al. 1986; Schechter et al. 1992). Insulin then begins to play a role in the regulation of food intake, acting as both a satiety hormone (Anika et al. 1980; VanderWeele 1994) and a trigger for food intake when present at elevated levels (Brandes 1977; Tordoff et al. 1982). Rats do not release insulin in response to the sweet taste of saccharin at birth, but this cephalic response is present by 21 days of age, and by 34 days, its magnitude is indistinguishable from that of adults (Bernstein and Woods 1980). Food intake in response to exogenously injected insulin first appears at about 25 days in rats (Lytle et al. 1971). However, exogenous insulin will induce hypoglycemia in rats younger than 10 days (Thompson et al. 1997), and in humans, neonatal hyperinsulinemia causes a hypoglycemia that requires clinical management (Thornton et al. 1993; Touati et al. 1998; Thomas 1999).

The presence of excessive circulating insulin early in development may have far-reaching consequences. In a previous study, we observed that giving rat pups daily insulin injections on postnatal days 9–20 attenuated the hypoglycemia produced by an insulin injection on postnatal day 60 (Thompson et al. 1997). When food was withheld for 2 h after injecting the 60 day olds with insulin (3 U/kg body weight), plasma glucose levels of control males dropped to 16 mg/dL, in contrast with only 67 mg/dL for males that had received insulin prior to weaning. Thus, insulin treatment during infancy significantly reduced the hypoglycemia that was induced by a severe insulin challenge during young adulthood.

The rat pups tested in our earlier study readily survived insulin that was injected at subcutaneous doses of 2 or 8 U/kg on postnatal days 9–20 (Thompson et al. 1997). With both doses, plasma glucose was below control levels at 2 h postinjection, and normoglycemia was regained by 6 h postinjection. Because the insulin injections lowered plasma glucose for less than 6 h daily, we became interested in examining how the effects of early insulin treatment might change if the daily exposure period were lengthened. This required that we first identify a method by which we could successfully deliver insulin to pups on a continuous basis.

In the present experiments, we compared the effects of delivering insulin to preweaning pups by three methods: daily injection, continuous-release pellet, and continuous-release osmotic minipump. A preliminary goal was to determine whether pups could survive continuously administered insulin as readily as they survived daily injections. Assuming that survival would occur, we wished to compare the effects of continuous and once-daily insulin administration on preweaning growth, plasma glucose and insulin levels, and (in Experiment 2) plasma triglycerides. Finally, we wished to compare the effects of injecting insulin on the plasma glucose, insulin, and triglyceride levels of pups and adults.

Experiment 1

In Experiment 1, pups received subcutaneous insulin for 10 days (postnatal days 9–18) via either a pellet designed to release insulin continuously (Innovative Research of America, Sarasota, Fla.), or a once-daily injection. Dependent variables were pup survival, growth rate, plasma glucose on treatment days 1, 8, and 10, and plasma insulin on treatment day 10.

Experiment 1 method

Animal care

The procedures used in Experiment 1, and in all subsequent experiments described in this paper, were approved by the Institutional Animal Care and Use Committee and followed the principles and guidelines of the Canadian Council on Animal Care (*Guide to the use and care of experimental animals*, 1984, 1993; http://www.ccac.ca/guides).

Subjects

Five 18-day pregnant Sprague–Dawley rats were obtained from Harlan Sprague Dawley (Indianapolis, Ind.). Each female, with her litter after parturition, was housed in a 20 × 12 × 10.5 in. glass cage (10-gal. aquarium) fitted with a screen top. In order to optimize ambient light, black construction paper covered the lower 80% of the cage's outer surface area and 25% of the screen top. Animals were provided with ad libitum tap water and food (Harlan Teklad Rat Chow, breeder's formula diet No. 7004). The laboratory was kept at an ambient temperature of 22°C with a 12 h light : 12 h dark cycle (lights on at 0700).

The day of parturition was designated postnatal day 0. On day 2, pups were counted and their sex determined. A total of 50 pups were born to the five dams, with litter sizes ranging from 8 to 13. Litters were equated at 10 pups on day 2 by cross-fostering. Sexes were balanced within litters insofar as possible; three litters contained five males and five females and two litters contained four males and six females.

Experiment 1 procedure

Treatment: postnatal days 9–18

On postnatal day 9, treatment groups were formed by assigning two pups from each litter (one male and one female whenever possible) to one of the five treatments shown in Table 1. Group 1 received, on postnatal days 9–18, a daily injection of 8 U bovine insulin/kg body weight mixed with physiological saline to a volume of 8 U insulin/mL solution. Group 2 received daily control injections of physiological saline in a volume of 1 mL/kg body weight. Group 3 was implanted subcutaneously on day 9 with a pellet designed to release 1.5 U of bovine insulin over a 12-day period (0.125 U insulin/day); the insulin was released as part of a matrix of cholesterol, lactose, celluloses, phosphates, and stearates. Group 4 was implanted with a pellet that released 0.5 U of insulin over a 12-day period (0.042 U/day). Group 5 was implanted with a control pellet, which released only the matrix.

The pellets were 3 mm in diameter and were implanted without anesthesia using an obturator to expel the pellet from a No. 10 trochar inserted subcutaneously between the shoulders. Injections were given using a 30-gauge needle inserted subcutaneously between the shoulders. Pellet implantation and daily injections always began at 1230, unless otherwise noted.

Table 1. Experiment 1 treatments and number of pups per group.

Group	10-day treatment on postnatal days 9–18	Number of pups (sex)
1	Injection: 8 U insulin/kg daily	10 (5 males, 5 females)
2	Injection: saline daily	10 (4 males, 6 females)
3	Pellet: 1.5 U, releases 0.125 U of insulin daily	10 (4 males, 6 females)
4	Pellet: 0.5 U, releases 0.042 U of insulin daily	10 (5 males, 5 females)
5	Pellet: control	10 (5 males, 5 females)

All pups were weighed daily, and their tails were marked with an indelible ink pen (Sharpie) to identify their sex and treatment group. Tail markings were augmented daily because mothers tended to lick the ink from their pups.

Rationale for insulin doses

Thompson et al. (1997) reported 100% survival for rat pups injected subcutaneously daily with 2 or 8 U insulin/kg body weight on postnatal days 9–20. The 8 U insulin/kg injection in the present study (group 1 in Table 1) thus replicated the highest dose used in the earlier study. The 1.5-U insulin pellet, which released 0.125 U of insulin daily (group 3), delivered a decreasing per kilogram dose as the pups gained weight. We estimated that pups would weigh about 21 g on day 9 and 46 g on day 18, so that throughout the 10-day treatment, the pellets would deliver a daily dose that fell within the 2- to 8-U/kg range injected in our earlier study. That is, a pellet releasing 0.125 U of insulin daily administers a 24-h dose of 6.0 U/kg to a pup weighing 0.021 kg on day 9 and 2.7 U/kg to a pup weighing 0.046 kg on day 18. We examined the effects of a smaller continuous dose with the 0.5-U insulin pellet (group 4), which released 0.042 U of insulin every 24 h. For pups of the weights described above, this pellet delivered 24-h doses of 2.0 U/kg on day 9 and 0.9 U/kg on day 18.

Plasma glucose on treatment days 1 and 8 (postnatal days 9 and 16)

On treatment days 1 and 8, plasma glucose was assessed 2 and 6 h after the treatment, which began at 1230 (for pellet groups, the treatment was weighing and pellet implantation on day 1 and weighing on day 8; for injection groups, the treatment was weighing and injection on days 1 and 8). The day's treatments were spaced at 2-min intervals between pups on both days, which allowed time for the interval between treatment and glucose testing to remain constant for all pups. To assess plasma glucose, litters were taken individually from the colony room to a nearby test room to minimize possible inter-litter communication that might alter the glucose levels of litters tested later. A drop of blood was obtained by removing the distal 1 mm of each pup's tail with a scalpel. Glucose concentrations were determined using a FastTake Compact glucose monitoring system (Lifescan; Johnson and Johnson, Milpitas, Calif.), which displays glucose levels falling between 20 and 600 mg/dL (1.1 and 33.3 mmol/L) 15 s after a drop of blood is applied. Two readings were obtained, and the mean of the readings was recorded. The duplicate assay procedure required approximately 60 s to complete for each pup.

Plasma glucose and insulin on treatment day 10 (postnatal day 18)

The amount of blood needed to assay plasma insulin was considerably greater than the single drop required for the FastTake glucose analysis. In order to obtain sufficient blood, the day 10 samples were collected by decapitation, a procedure that required 2–3 min per animal. This time requirement was too lengthy to allow blood to be collected 2 h after weighing and injection, as had occurred on treatment days 1 and 8, since it required 2.5 h to decapitate all of the pups. Therefore, the plasma constituents on treatment day 10 were assessed 3 h posttreatment. On this day, the weighing and injection procedures were begun at 1000, with a delay of 3 min between animals, and blood collection began at 1300 hours. A small portion of the blood was used to assay plasma glucose using the FastTake monitor. The remainder of the blood was collected in heparinized tubes, centrifuged, and the plasma removed and frozen at –100°C until it was assayed for insulin. Plasma insulin concentrations were determined by radioimmunoassay using a commercial RIA kit (Immuchem^{TM} Coated Tube Insulin RIA; ICN Pharmaceuticals, Inc., Costa Mesa, Calif.). Duplicate assays were run on each plasma sample and the average was determined and recorded.

Experiment 1 results

Mortality

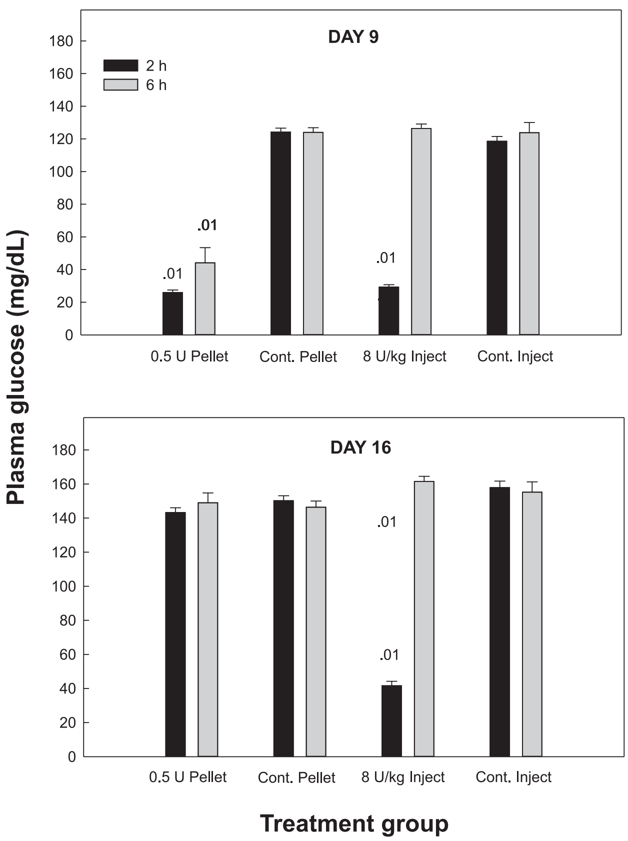
Twelve of the 50 pups failed to survive 24 h after treatment on day 9. All 12 of these animals had received a pellet containing insulin; no pups with a control pellet died. Deaths occurred for nine of 10 pups with a 1.5-U insulin pellet and for three of 10 pups with a 0.5 U pellet. No deaths occurred on treatment days 2–10.

The high mortality produced by the insulin pellets occurred despite the fact that both pellet sizes were designed to deliver less than 8 U insulin/kg over 24 h, an amount that was well tolerated by pups given this dose in a single injection. Table 2 shows the average body weight on day 9 for the pups who survived each treatment as well as the total units of insulin received per kilogram of body weight during the 24 h immediately following implantation on day 9. The single pup who survived a 1.5-U insulin pellet received 6.3 U insulin/kg over the 24-h period, and the seven pups who survived a 0.5-U insulin pellet received an average of 1.9 U insulin/kg. The ability to survive the pellets was not closely related to body weight. The nine pups who failed to survive a 1.5-U insulin pellet weighed an average of 21.77 ± 0.44 g on day 9, giving them 5.7 U insulin/kg over 24 h (compared with 6.3 U/kg for the pup who survived). The three pups who failed to survive a 0.5-U pellet weighed an average of 21.71 ± 0.74 g on day 9, giving them 1.9 U insulin/kg over 24 h (compared with 1.9 U/kg for the pups who survived). As can be seen in Table 2, there were no sex differences in the ability to survive the insulin pellets.

Table 2. Number of survivors (from groups of 10) in Experiment 1 and mean (±SE) body weight and 24-h insulin dose on postnatal day 9.

Treatment	No. of survivors (sex)	Mean body weight (g)	Mean insulin dose (U/kg body weight)
12-day pellet: 0.5 U of insulin	7 (3 males, 4 females)	21.83±0.76	1.9
12-day pellet: 1.5 U of insulin	1 (0 males, 1 female)	19.75±0.00	6.3
12-day pellet: control	10 (5 males, 5 females)	22.37±0.87	—
Injection: 8 U insulin/kg	10 (5 males, 5 females)	22.31±0.54	8.0
Injection: saline	10 (5 males, 5 females)	21.58±0.53	—

Fig. 1. Mean plasma glucose (±SE) for pups in Experiment 1. Pups with a 0.5-U pellet (0.125 U of insulin daily or about 1.9 U/kg on postnatal day 9) were hypoglycemic on day 9 but not on day 16. Pups injected with 8 U insulin/kg were hypoglycemic at 2 h postinjection on days 9 and 16 and were normoglycemic at 6 h postinjection on both days.



Data analysis

Data were analyzed using analysis of variance (ANOVA) procedures conducted with the BMDP 2V statistical program (release 7.0). This program allows factors involving repeated measures (e.g., days of age) to be divided into components, permitting orthogonal examinations of linear and nonlinear trends in the data. All data are reported as means ± SE. Probabilities ≤0.05 were considered statistically significant. Post hoc comparisons of means were assessed using the Newman–Keuls procedure.

Because only one pup survived the 1.5-U insulin pellet treatment, it was impossible to determine variances for this treatment group. Therefore, the ANOVAs reported for Experiment 1 compare only the four groups (37 pups) where multiple animals survived (i.e., Groups 1, 2, 4, and 5 in Table 1). Any data reported for the lone survivor of the 1.5-U insulin pellet are specifically identified and are not included in any analysis.

Plasma glucose on treatment days 1 and 8 (postnatal days 9 and 16)

Differences in plasma glucose were analyzed using a four-factor ANOVA, with the four treatments (0.5 U insulin pellet, control pellet, 8 U/kg insulin injection, control injection) and sex as between-group variables and treatment days (1 and 8) and hours (assays 2 and 6 h after treatment) as within-group variables. There was a significant treatments × days × hours interaction ($F_{[3,29]} = 234.59$, $p < 0.0001$), which is shown in Fig. 1. To facilitate interpretation, the interaction is described first for differences that occurred on treatment day 1 and then for differences that occurred on treatment day 8 (see below).

Plasma glucose on treatment day 1 (Fig. 1, top panel)

Two hours after implantation on postnatal day 9, pups that had received a 0.5-U insulin pellet were hypoglycemic relative to pups that received a control pellet (26.0 ± 1.5 versus 124.2 ± 2.4 mg/dL, $p < 0.01$). By 6 h postimplantation, the glucose levels of pups in the 0.5-U pellet group had increased slightly (to 44.1 ± 9.3 mg/dL, $p < 0.01$), although these animals continued to be hypoglycemic relative to pups with a control pellet (124.0 ± 2.8 mg/dL, $p < 0.01$). Pups injected with 8 U insulin/kg were hypoglycemic after 2 h relative to pups that received a control injection (29.3 ± 1.5 versus 118.6 ± 2.9 mg/dL, $p < 0.01$), and their 2-h glucose levels did not differ significantly from those of pups who had received a 0.5-U insulin pellet. By 6 h, however, the plasma glucose of pups injected with 8 U insulin/kg had increased to 126.4 ± 2.7 mg/dL ($p < 0.01$), a level that did not differ from that of the pups given control injections (123.8 ± 6.2 mg/dL). There were no differences in plasma glucose between pups given control pellets and pups given control injections at either 2 or 6 h.

On treatment day 1, the plasma glucose of the single survivor with a 1.5-U insulin pellet was 24 mg/dL at 2 h postimplantation, and at 6 h, it was below 20 mg/dL (the minimum the apparatus could record).

Plasma glucose on treatment day 8 (Fig. 1, bottom panel)

The plasma glucose levels of pups with a 0.5-U insulin pellet did not differ from those of pups with a control pellet on postnatal day 16 at either 2 or 6 h after weighing. Thus, pups with a 0.5-U insulin pellet were no longer hypoglycemic by treatment day 8. In contrast, pups injected with 8 U insulin/kg exhibited a period of hypoglycemia on treatment day 8 that was very similar to that seen on day 1; their glucose levels were lower than those of pups given a control injection at 2 h postinjection (41.6 ± 2.6 and 157.9 ± 3.8 mg/dL, respectively, $p < 0.01$) but not at 6 h (161.5 ± 3.0 and 155.2 ± 6.1 mg/dL, respectively).

On treatment day 8, the plasma glucose of the single survivor with a 1.5-U insulin pellet was 144 mg/dL at 2 h after weighing and 126 mg/dL at 6 h.

Plasma glucose on treatment day 10 (postnatal day 18)

Differences in plasma glucose on treatment day 10 were assessed separately from those on days 1 and 8 because the 3-h treatment–assay interval required on day 10 differed from the 2- and 6-h intervals used on days 1 and 8. The ANOVA for day 10 contained two factors, with the four treatments and sex as between-group variables. This analysis revealed no significant group differences, with an average glucose level of 147.64 ± 5.5 mg/dL. However, the glucose levels of pups that had been injected with 8 U insulin/kg 3 h earlier were quite variable, ranging from 48 to 196 mg/dL. This suggests that there were individual differences in the rate that normoglycemia was regained after the insulin injection.

Plasma insulin on treatment day 10 (postnatal day 18)

Differences in plasma insulin on treatment day 10 were analyzed using a two-factor ANOVA identical to that used to assess differences in plasma glucose (see above). There were no significant differences in plasma insulin due to sex or treatment. The average plasma insulin level of pups who had been injected 3 h earlier with 8 U insulin/kg was 42.7 ± 6.7 μU/mL compared with 36.2 ± 4.9 μU/mL for pups injected with saline. The average insulin level of pups with a 0.5-U insulin pellet was 30.3 ± 3.7 μU/mL compared with 33.0 ± 3.2 μU/mL for pups with a control pellet.

Body weight on treatment days 1–10 (postnatal days 9–18)

Body weights during the treatment period were evaluated using an *S*(treatment × sex) × days ANOVA, with the four treatments and sex as between-group variables and the 10 treatment days as a repeated measure. There were no differences in weight gain due to treatment or sex. The overall days effect was significant ($F_{[8,232]} = 1685.62$, $p < 0.0001$) as was the linear component of this effect ($F_{[1,232]} = 2{,}705.77$, $p < 0.0001$). Mean body weights across the 10 treatment days for all pups combined are shown in Table 3. As is evident from the table, body weights averaged 22.04 ± 0.33 g on postnatal day 9 and 46.70 ± 0.66 g on day 18, indicating an average daily gain of 2.74 g during the treatment period.

Table 3. Mean (± SE) body weight on postnatal days 9–18 (treatment days 1–10) for pups in the four treatment groups of Experiment 1.

Postnatal day	Mean body weight (g)
9	22.04 (0.33)
10	24.83 (0.34)
11	27.74 (0.39)
12	30.64 (0.41)
13	33.12 (0.47)
14	35.90 (0.52)
15	38.03 (0.58)
16	40.49 (0.62)
17	43.01 (0.62)
18	46.70 (0.66)

Experiment 1 discussion

Insulin that was injected daily at a dose of 8 U/kg caused no deaths in this study, either on the initial day of treatment or on any subsequent day. This replicates our earlier observation that rat pups 9–20 days old readily survive exogenous insulin that is injected in a single daily dose of 8 U/kg body weight (Thompson et al. 1997). In contrast, many pups died within 24 h of receiving a pellet that released insulin continuously, even though the total amount released over the 24 h was less than 8 U/kg. The 1.5-U insulin pellet delivered an average of 5.7 U insulin/kg body weight on treatment day 1, and it caused death for 90% of the pups who received it. The smaller 0.5-U pellet delivered an average of only 1.9 U/kg on day 1, but even this low dose caused death within 24 h for 30% of the pups who received it.

It is not certain why an insulin dose that was readily survived when administered by injection was fatal when administered by pellet. It is unlikely that the pellet-induced deaths can be attributed to stress accompanying the implantation procedure, since no deaths occurred in animals implanted with control pellets. It is possible that the duration of insulin exposure is critical. On treatment day 1, pups that received a 0.5-U pellet did not regain normal blood sugar levels during the first 6 h after pellet implantation, whereas pups who were injected with 8 U insulin/kg were normoglycemic by 6 h postinjection (see Fig. 1). Thus, injected animals were normoglycemic for at least 18 h prior to the next day's injection, whereas it is possible that pups with pellets did not regain normoglycemia during the first 24 h. This raises the question whether 9-day-old rats may tolerate an acute period of severe hyperinsulinemia more readily than they tolerate a hyperinsulinemic episode that is initially less intense but that lasts longer.

Despite the substantial hypoglycemia produced by the 0.5-U insulin pellets on treatment day 1 (postnatal day 9), the pups who survived this treatment had plasma glucose levels that did not differ from control levels on treatment days 8 and 10. The present data do not allow a definitive explanation for this observation, but several possibilities exist. First of all, because pups doubled their body weight during the 10-day treatment period, pups with pellets received a per kilogram insulin dose on treatment day 10 that was half what they had received on day 1, whereas injected animals received the same insulin dose of 8 U/kg on all 10 treatment days. Another possibility, although one we consider unlikely, is that the insulin pellets were no longer releasing insulin at the end of the study. Clearly, the pellets were releasing insulin on treatment day 1. We cannot verify that insulin release continued throughout the entire 10-day treatment period, and we recognize that on treatment day 10, the plasma insulin levels of pups with insulin pellets did not differ from those of pups with control pellets. However, other investigators have reported that adult rats exhibit normal plasma insulin at the end of a 14-day period where animals receive up to 5 U of insulin daily via an osmotic minipump (Destefano et al.

1991). Thus, we assume that the pellets, which are marketed as releasing insulin at a constant rate for 12 days, remained fully active on treatment day 10. An alternative possibility is that the exogenous insulin induced a compensatory lowering of endogenous insulin production or an increase in insulin degradation.

A down-regulation of insulin receptors also may have contributed to the normoglycemia observed by the eighth treatment day in pups with insulin pellets. In vitro evidence indicates that a down-regulation of fetal lung insulin receptors occurs when lung explants are exposed to exogenous insulin (Zmora et al. 1992). Moreover, the brains of adult cats have abnormally low numbers of benzodiazepine receptors if their mothers are exposed to diazepam during pregnancy (Marczynski and Urbancic 1988). Further work is needed to clarify whether a down-regulation of insulin receptors occurs when rat pups are chronically exposed to high levels of insulin.

Chronic exposure to exogenous insulin had no effect on the body weight of pups in this study. Pups given insulin grew at control levels throughout the 10-day treatment period, regardless of whether the insulin was delivered via pellet or injection. This suggests that the pups did not increase their food consumption in response to insulin, which is in accord with the finding that exogenous insulin does not trigger food intake in rats prior to postnatal day 25 (Lytle et al. 1971).

Experiment 2

Experiment 1 raised several questions. One was whether pups receiving continuous insulin might cease exhibiting hypoglycemia more quickly than pups receiving daily injections if both groups received the same daily per kilogram doses of insulin. A second was whether pups might survive continuous insulin more readily if it were administered by osmotic minipump rather than by pellet.

Experiment 2 had two parts. In Experiment 2A, 9-day-old pups were given the same daily dose of insulin or control solution for 10 days via either an injection or an osmotic minipump, and plasma glucose, insulin, and triglyceride levels were compared. In Experiment 2B, possible age differences in the metabolic response to insulin were investigated. Pups and adults were injected daily for 10 days with the same per kilogram dose of insulin, and plasma glucose, insulin, and triglyceride levels were compared. Triglyceride analyses, which were not performed in Experiment 1, were included in Experiment 2 to provide a second insulin-sensitive indicator of metabolic state.

Experiment 2 method

Pup subjects

Eight 17-day-pregnant Sprague–Dawley rats were obtained from Harlan Sprague Dawley (Indianapolis, Ind.). These were housed and fed as in Experiment 1. A total of 87 pups (47 males and 40 females) were born to the eight dams, with litter sizes ranging from 8 to 11. The day of parturition was designated postnatal day 0. Pups were counted, sexed, and cross-fostered as necessary on postnatal day 2 to equate litters sizes at 10 pups each, with five males and five females per litter. The only exception was that one dam reared an extra male due to a miscount. Thus, 41 male and 40 female pups were treated.

Adult subjects

Thirty-three adult Sprague–Dawley rats (17 male and 16 female) aged 52 days were obtained from Harlan Sprague Dawley. Rats were housed individually in standard wire cages. They were acclimated to handling by holding them for several seconds and placing them in the weighing cage each day for 1 week prior to treatment onset. Animals were provided with ad libitum tap water and food (Harlan Teklad rodent diet No. 8604). The laboratory environment was as described in Experiment 1.

Experiment 2 procedure

Pup treatments: postnatal days 9–18

On postnatal day 9, pups were assigned to one of the five treatments shown at the top of Table 4. Groups 1, 2, and 3 received a daily subcutaneous injection for 10 days, beginning on postnatal day 9, with the injection procedure as described in Experiment 1. Group 1 received 8 U bovine insulin/kg mixed with physiological saline to a volume of 8 U/mL. Group 2 received 0.184 U of bovine insulin. Group 3 received physiological saline in a volume of 1 mL/kg body weight. Groups 4 and 5 carried a subcutaneous osmotic minipump (Alzet No. 1002, Alza Corp., Palo Alto, Calif.) from postnatal days 9 to 18. For group 4, the minipump delivered 0.184 U of bovine saline daily, and for group 5, it delivered saline. One male and one female from each litter were assigned to each of the five treatments, making 16 pups per treatment; the extra male was assigned to group 1 (8 U insulin/kg injection). Except where noted otherwise, all pups were weighed daily between 1300 and 1400.

When implanted subcutaneously, the Alzet No. 1002 osmotic minipump is recommended for animals weighing at least 10 g. It delivers 0.24 μL solution/h (5.76 μL daily) for up to 14 days. The minipumps for the pups in Group 4 were filled with insulin in a solution of 0.032 U insulin/μL saline, so that the minipumps delivered 0.184 U of insulin every 24 h.

Rationale for pup insulin doses

The 8 U insulin/kg dose injected in group 1 duplicated a dose that was tolerated well by pups in a previous study (Thompson et al. 1997) and by the pups in Experiment 1 of the present study. We estimated that pups would weigh up to 23 g on day 9 and 46 g by day 18. The daily minipump release of 0.184 U of insulin for the animals in group 4 thus yielded a 24-h dose of approximately 8.0 U/kg on postnatal day 9 and 4.0 U/kg on day 18. This daily minipump dose was higher than the 0.125-U dose released daily by the larger of the two insulin pellets in Experiment 1, which 90% of pups failed to survive. Nevertheless, we felt confident that this dose would be tolerated because pilot work in our laboratory had suggested that pups survive insulin delivered by minipump more readily than they survive insulin delivered by pellet. Assuming that survival would occur, we wished the 24-h minipump dose on day 9 to be comparable with the 8 U/kg dose that was given by injection to the pups in group 1. The pups in group 2 were injected daily with 0.184 U of insulin in order to directly compare the effects of receiving

Table 4. Experiment 2 treatments for pups and adults and number of animals per group.

Group	Treatment	Number (sex)
Pups	10-day treatment on postnatal days 9–18	
1	Injection: 8 U insulin/kg daily	17 (9 males, 8 females)
2	Injection: 0.184 U of insulin daily (8 U/kg on day 9)	16 (8 males, 8 females)
3	Injection: saline daily	16 (8 males, 8 females)
4	Minipump: 0.184 U of insulin daily (8 U/kg on day 9)	16 (8 males, 8 females)
5	Minipump: saline	16 (8 males, 8 females)
Adults	10-day treatment on postnatal days 60–69	
6	Injection: 8 U insulin/kg daily	17 (9 males, 8 females)
7	Injection: saline daily	16 (8 males, 8 females)

by injection or minipump the same daily dose of insulin for 10 days.

Adult treatments: postnatal days 60–69

On postnatal day 60, treatment groups were formed by assigning the 33 adults to one of the two treatments shown in the bottom part of Table 4 (i.e., groups 6 and 7). Group 6 received a daily subcutaneous injection of bovine insulin in a dose of 8 U/kg and group 7 received a daily injection of saline. Injection mixtures and procedures were identical to those described above for the pups in groups 1 and 3. Eight males and eight females were assigned to each treatment, with the extra male assigned to group 6 (inject 8 U insulin/kg). Daily injections occurred immediately after the animals were weighed.

Minipump implantation in pups

Hypothermia is safe, and it has been shown to be an effective technique for inducing anesthesia in neonatal and preweaning rats (Phifer and Terry 1986; Danneman and Mandrell 1997). We therefore used moderate hypothermia to subcutaneously implant the osmotic minipumps in this study. On postnatal day 9, pups in groups 4 and 5 were immersed to their necks in ice water for 3 min. Immediately after withdrawal from the ice water, the pups' backs were swabbed with 70% ethanol. A 1-cm longitudinal incision then was made with a scalpel along the skin of the back, immediately behind the shoulder blades. A closed sterile hemostat was inserted into the incision and opened to create a cavity for the sterile minipump, which was inserted with the insulin-releasing end toward the pup's head. The incision was stapled with wound clips and covered with Vetbond surgical adhesive (3 M Animal Care Products, St. Paul, Minn.), a procedure that decreases postsurgical tissue damage induced through normal maternal care in pups with subcutaneous minipumps (Thornton and Smith 1997). It took approximately 1.5 min to implant the minipump in each pup, and pups were returned to their mothers about 15 min later. Mothers readily accepted all pups after surgery. The minipumps appeared to be well tolerated and caused no obvious difficulty with nursing.

Plasma glucose assays on treatment days 2, 5, and 8

Pups: postnatal days 10, 13, and 16

Plasma glucose levels were assessed on treatment days 2, 5, and 8 with a FastTake glucose monitor, as described in Experiment 1. Assays were taken both 2 and 4 h after weighing and injection for injected animals and after weighing for the others. Approximately 1 mm of the end of the pup's tail was removed with a scalpel to enable blood collection. Two glucose readings were taken for each pup. The average was recorded if the two readings were within 20 mg/dL of each other; a third reading was taken if the first two differed by more than 20 mg/dL.

Adults: postnatal days 61, 64, and 67

On treatment days 2, 5, and 8, plasma glucose levels were measured with a FastTake glucose monitor 2 and 4 h after the daily injection, as described for the pups.

Plasma glucose, insulin, and triglyceride assays on treatment day 10

Pups: postnatal day 18

The amount of blood required to assay plasma insulin, triglycerides, and glucose on treatment day 10 was considerably greater than that required for the FastTake glucose analyses performed on days 2, 5, and 8. Therefore, blood samples on treatment day 10 were collected by decapitation, a procedure that required 2–3 min per animal. Decapitation occurred 4 h after weighing (and injection for injected pups). On this day, the weighing and injection treatments began at 0900, with a 2.5-min delay between animals. This delay permitted blood collection to begin at 1300, with a 4-h interval between treatment and blood collection for each of the 81 pups. Blood was collected in heparinized tubes, centrifuged, and the plasma removed and frozen at –100°C until analysis.

Because the 2.5-min between-animal time interval needed to decapitate 81 animals in 4 h did not allow sufficient time to include a FastTake glucose analysis, plasma glucose concentrations on day 10 were not assessed by the FastTake monitor; rather, they were determined spectrophotometrically by a coupled glucose oxidase, peroxidase procedure (Sigma Chemical Co., St. Louis, Mo.). Plasma insulin concentrations were determined by radioimmunoassay, as described in Experiment 1. Plasma triglyceride concentrations were determined spectrophotometrically using a coupled glycerol kinase, glycerylphosphate oxidase, peroxidase assay (Liquicolor®, Stanbio Laboratories Inc., San Antonio, Tex.). For all procedures, duplicate assays were run on each plasma sample and the average was determined and recorded.

For pups with osmotic minipumps, the minipump was removed from each animal at the time of decapitation. To verify the release of insulin or saline, the solution remaining in each minipump was removed and measured by a microliter syringe fitted with a 26-gauge needle (Hamilton). In all cases, the amount of solution remaining in each minipump was in a range that indicated that the minipump had released the appropriate volume of solution during the 10-day treatment period.

Adults: postnatal day 69

On treatment day 10, the adults were sacrificed by cervical dislocation and 1.0–2.5 mL of blood was obtained from each animal by cardiac puncture. On this day, weighing and injection treatments began at 0900, with a 6-min delay between animals. This permitted blood collection to begin at 1300, with a 4-h interval between injection and blood collection for each of the 33 adults. The blood was collected in heparinized tubes, centrifuged, and the plasma removed and frozen at –100°C until assayed to determine plasma glucose, insulin, and triglyceride concentrations. Assay procedures were as described above for pups.

Experiment 2 data analysis

Data were analyzed by ANOVA using the BMDP 2V statistical program described in Experiment 1. All data are reported as means ± SE. Probabilities ≤0.05 were considered statistically significant, and post hoc comparisons were made using the Newman–Keuls procedure. The following two sets of analyses were conducted.

Pup injection versus pup minipump

These analyses, referred to hereafter as Experiment 2A, compared data from four of the five groups of pups (groups 2–5 in Table 4). These pups either had received a daily injection (groups 2 and 3) or had been implanted with a minipump (groups 4 and 5). Both administration routes delivered an identical dose of 0.184 U of either insulin or saline daily. Calculated over a 24-h period, the insulin dose was approximately 8 U/kg on postnatal day 9 and 4 U/kg on day 18.

Infant injection versus adult injection

These analyses, referred to hereafter as Experiment 2B, compared data from the pups and adults who were injected daily with 8 U insulin/kg (groups 1 and 6 in Table 4) or with a saline control solution (groups 3 and 7). The data from the saline-injected control pups were used in both the Experiment 2A and 2B analyses. All other treatment groups were independent in the two sets of analyses.

Experiment 2A results: pup injection versus pup minipump

Plasma glucose on treatment days 2, 5, and 8

Differences in plasma glucose were analyzed with an *S*(treatments × sex) × treatment days × hours ANOVA. There were four treatments (insulin or saline injection, insulin or saline minipump), two sexes, three treatment days (glucose measured on second, fifth, and eighth day of treatment), and two hours (glucose measured 2 and 4 h after the daily injection and (or) weighing). Treatments and sex were between-group measures, and treatment days and hours were repeated measures.

The treatments × treatment days × hours interaction was significant ($F_{[6,112]} = 8.26$, $p < 0.0001$), and this three-way interaction is shown in Fig. 2. Looking first at the 2-h posttreatment data (left side of Fig. 2), it is evident that plasma glucose levels were lower for pups injected with insulin than they were for pups injected with saline, and the magnitude of this difference was stable across the second, fifth, and eighth treatment days (overall means were 32.81 mg/dL after insulin and 134.71 mg/dL after saline). In contrast, the glucose levels of pups with an insulin minipump were lower than those of pups with a saline minipump only on the second treatment day (83.88 and 131.06 mg/dL, respectively). Glucose levels were stable on the fifth and eighth treatment days, and the levels of pups with insulin and saline minipumps did not differ (overall means were 128.94 and 137.78 mg/dL, respectively). Accordingly, pups with insulin minipumps exhibited a significant increase in glucose levels between the second and fifth treatment days.

At 4 h postinjection (right side of Fig. 2), pups with insulin and saline minipumps exhibited plasma glucose levels similar to those seen at 2 h, so that pups with insulin minipumps had lower glucose levels than pups with saline minipumps on the second treatment day but not on the fifth or eighth day. For pups injected with saline, glucose levels also were similar at 2 and 4 h postinjection. For pups injected with insulin, however, plasma glucose levels were considerably higher at 4 h postinjection than at 2 h. On the second treatment day, the 4-h glucose levels of pups injected with insulin were equal to saline control values, and they became increasingly higher than control values on the fifth and eighth treatment days.

Plasma glucose on treatment day 10: pup injection versus pup minipump

Plasma glucose values on treatment day 10 were not directly compared with those obtained on days 2, 5, and 8 because of the different assay procedures used. Therefore, group differences in plasma glucose on treatment day 10 were analyzed separately with an *S*(treatments × sex) ANOVA. There were four treatments (insulin injection, saline injection, insulin minipump, or saline minipump) and two sexes, and both factors were between-group measures.

The treatments main effect was statistically significant ($F_{[3,56]} = 5.01$, $p < 0.01$). Pups injected with insulin 4 h earlier had a mean plasma glucose level that was significantly higher than that of the pups injected with saline (185.81 ± 3.23 and 175.44 ± 2.90 mg/dL, respectively, $p < 0.05$); this hyperglycemia was similar to that exhibited by these insulin-injected pups at 4 h postinjection on treatment days 5 and 8 (see above). The 4-h glucose levels of pups injected with insulin also were higher than those of pups with insulin minipumps (173.12 ± 1.65 mg/dL) or saline minipumps (174.00 ± 2.60 mg/dL), whose glucose levels did not differ. Thus, daily insulin injections raised plasma glucose at 4 h postinjection, whereas insulin given via a minipump did not raise plasma glucose above control levels.

Plasma triglycerides on treatment day 10: pup injection versus pup minipump

Between-group differences in plasma triglycerides were analyzed with an *S*(treatments × sex) ANOVA identical to that used to analyze plasma glucose on treatment day 10 (see above). The treatments main effect was statistically significant ($F_{[3,56]} = 8.19$, $p < 0.001$). Figure 3 shows that injecting 0.184 U of insulin 4 h earlier lowered plasma triglycerides

Fig. 2. Mean plasma glucose (±SE) for pups in Experiment 2A. At 2 h postinjection, pups injected with 0.184 U of insulin daily (8 U/kg on day 9) were hypoglycemic on treatment days 2, 5, and 8. At 4 h, they were normoglycemic on day 2 and hyperglycemic on days 5 and 8. Pups who received 0.184 U of insulin daily via a minipump were hypoglycemic on day 2 and normoglycemic on days 5 and 8.

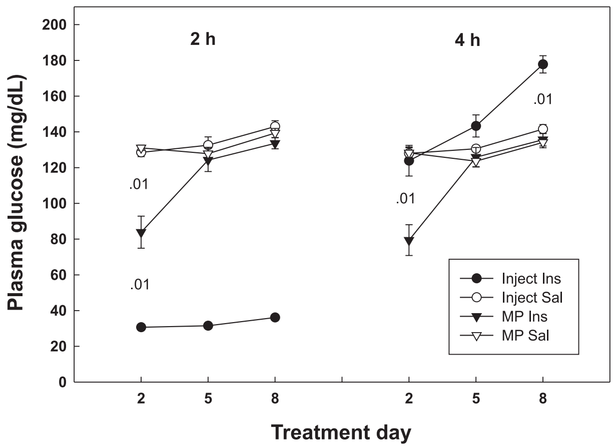
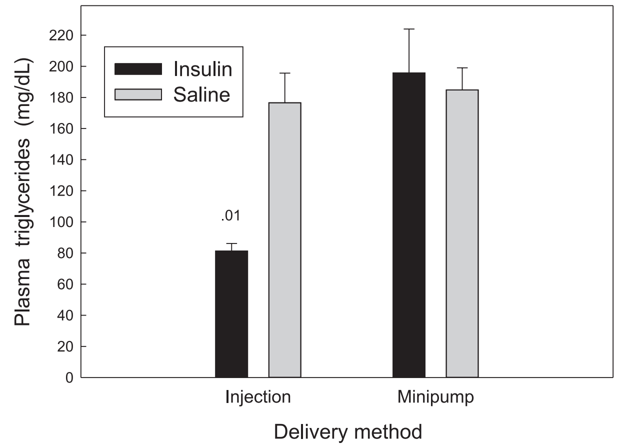


Fig. 3. Mean plasma triglycerides (±SE) on treatment day 10 for pups in Experiment 2A. Triglycerides fell below control levels for pups injected 4 h earlier with 0.184 U of insulin but were equal to control levels for pups given 0.184 U of insulin daily via a minipump.



to a level that was significantly below that seen in saline-injected pups (81.31 ± 4.84 versus 176.56 ± 19.05 mg/dL, respectively). In contrast, the triglyceride levels of pups given insulin via minipump (195 ± 28.22 mg/dL) did not differ significantly from the levels of pups with a saline minipump (184.81 ± 14.29 mg/dL), and both minipump groups had significantly higher triglyceride levels than did pups given insulin by injection.

Plasma insulin on treatment day 10: pup injection versus pup minipump

Between-group differences in plasma insulin were analyzed with an *S*(treatments × sex) ANOVA identical to that used to analyze plasma glucose on treatment day 10 (see above). Neither the treatments nor sex effect was statistically significant. Pups injected with insulin 4 h earlier had a plasma insulin level of 48.54 ± 4.24 μU/mL, pups injected with saline had a level of 42.11 ± 3.71 μU/mL, pups with an insulin minipump had a level of 37.00 ± 2.62 μU/mL, and pups with a saline minipump had a level of 45.89 ± 4.35 μU/mL.

Body weight: pup injection versus pup minipump

Differences in body weight were analyzed using an *S*(treatments × sex) × treatment days ANOVA. There were four treatments (insulin injection, saline injection, insulin minipump, or saline minipump), two sexes, and 10 treatment days (weight measured on the first through 10th days of treatment). Treatments and sex were between-group measures, and treatment days was a repeated measure.

The treatments × treatment days interaction was statistically significant ($F_{[27,504]} = 2.79$, $p < 0.0001$), and the quadratic (across treatment days) component of this interaction also was significant ($F_{[3,56]} = 7.19$, $p < 0.001$). The two-way interaction is shown in Fig. 4. It is apparent from Fig. 4 that pups injected with saline gained weight faster than did pups in the other three groups during the first few treatment days, whereas pups injected with insulin gained weight faster than did pups in any other group during treatment days 7–10.

Experiment 2B results: pup injection versus adult injection

Plasma glucose on treatment days 2, 5, and 8

Differences in plasma glucose were analyzed with an *S*(treatments × ages × sex) × test days × hours ANOVA. There were two treatments (injected with saline or 8 U insulin/kg), two ages (treatments began on postnatal day 9 or 60), two sexes, three test days (glucose measured on the second, fifth, and eighth days of treatment), and two hours (glucose measured 2 and 4 h after the daily injection). Treatments, ages, and sex were between-group measures, and test days and hours were repeated measures.

The treatments × ages × test days × hours interaction was significant ($F_{[2,116]} = 12.02$, $p < 0.0001$). The linear (across test days) component of this interaction also was significant ($F_{[1,58]} = 76.51$, $p < 0.001$) as was the quadratic component ($F_{[1,58]} = 7.24$, $p < 0.01$). This interaction is shown in Fig. 5. The left side of Fig. 5 shows differences among treatment groups, and across days, at 2 h postinjection. At 2 h, it is apparent that injecting 8 U insulin/kg lowered plasma glucose to a greater degree in pups than in adults (to 31.61 ± 0.71 and 50.84 ± 2.49 mg/dL, respectively). After a saline injection, on the other hand, plasma glucose levels were higher in pups than in adults (134.71 ± 2.47 and 105.19 ± 1.17 mg/dL, respectively). Only the pups injected with saline exhibited any change across test days at 2 h postinjection; for these animals, plasma glucose levels were lower on the second treatment day than on the eighth day (128.56 ± 2.39 and 143.00 ± 3.28 mg/dL, respectively).

The right side of Fig. 5 shows differences among treatment groups, and across days, at 4 h postinjection. Four hours after a saline injection, the plasma glucose levels of adults were consistently lower than those of pups, as they had been at 2 h. Four hours after an insulin injection, neither the adults nor the pups were ever hypoglycemic. In fact, the adults injected with insulin were hyperglycemic on all three

Fig. 4. Mean body weights (±SE) for pups in Experiment 2A. Pups given saline injections grew faster during the treatment period than did pups given saline via a minipump. Saline-injected pups initially grew faster than pups given a daily injection of 0.184 U of insulin, but the weight difference disappeared by treatment day 9.

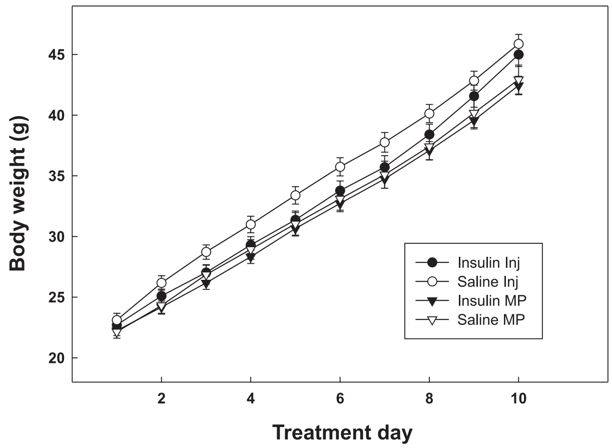
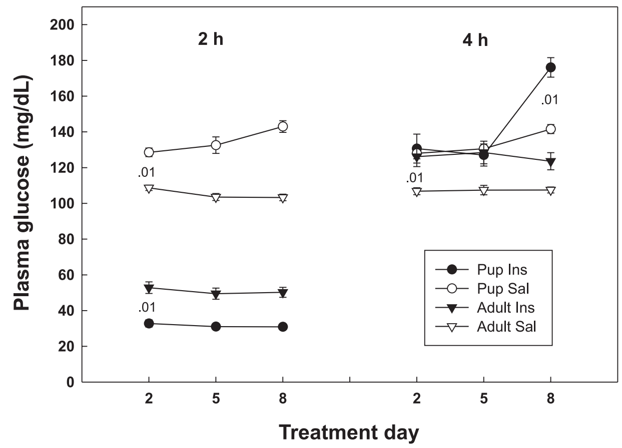


Fig. 5. Mean plasma glucose (±SE) for pups and adults injected daily with 0 or 8 U insulin/kg in Experiment 2B. Glucose levels were higher in pups than in adults 2 h after a saline injection and lower in pups than in adults after an insulin injection. Four hours after an insulin injection, adults were hyperglycemic relative to controls on treatment days 2, 5, and 8, while pups were hyperglycemic only on day 8.



treatment days relative to saline-injected controls (overall means of 126.06 ± 4.69 versus 107.25 ± 1.76 mg/dL, respectively). For pups, glucose levels 4 h after an insulin or saline injection were equivalent on treatment days 2 and 5 (overall means of 128.85 ± 6.16 and 129.30 ± 1.78 mg/dL, respectively), but pups became hyperglycemic relative to controls on treatment day 8 (176.06 ± 5.45 versus 141.56 ± 2.51 mg/dL, respectively). Thus, adults and pups both became hyperglycemic 4 h after an insulin injection, but pups required several days longer for this overcompensatory rebound to appear.

Plasma glucose on treatment day 10: pup injection versus adult injection

As in Experiment 2A, the plasma glucose values recorded on treatment day 10 were not directly compared with those obtained on days 2, 5, and 8 because of the different assay procedures used. Rather, differences in plasma glucose on treatment day 10 were analyzed separately with an *S*(treatments × sex × ages) ANOVA. There were two treatments (insulin or saline injection), two sexes, and two ages (treatments begun on postnatal day 9 or 60). All three factors were between-group measures.

The treatments effect was statistically significant ($F_{[1,58]}$ = 33.08, $p < 0.0001$). Plasma glucose levels were higher in pups and adults 4 h after an insulin injection (178.95 ± 5.27 mg/dL) than they were in animals that had been injected with saline (151.75 ± 4.57 mg/dL).

The age effect also was significant ($F_{[1,58]}$ = 81.62, $p <$ 0.0001). Regardless of whether they had been injected with insulin or saline, plasma glucose levels were higher in pups (186.71 ± 3.35 mg/dL) than in adults (143.99 ± 4.63 mg/dL).

Plasma triglycerides on treatment day 10: pup injection versus adult injection

Between-group differences in triglyceride levels 4 h after injection on treatment day 10 were analyzed with an *S*(treatments × ages × sex) ANOVA identical to that used to assess plasma glucose on treatment day 10 (see above). The treatments × ages interaction was significant ($F_{[1,58]}$ = 18.85, $p <$ 0.01), and this interaction is shown in Fig. 6. It is clear that plasma triglycerides were higher for pups injected with saline (176.56 ± 19.l0 mg/dL) than they were for pups injected with insulin (86.28 ± 6.18 mg/dL, $p < 0.01$). In turn, pups injected with insulin had higher triglyceride levels than did adults injected with either insulin (67.76 ± 4.33 mg/dL, $p <$ 0.05) or saline (71.81 ± 4.28 mg/dL, $p < 0.05$). The two adult groups did not differ from each other. Thus, triglyceride levels were higher in pups than in adults regardless of the substance injected, but the magnitude of the difference was greatest for pups injected with saline.

Plasma insulin on treatment day 10: pup injection versus adult injection

Between-group differences in plasma insulin 4 h postinjection on treatment day 10 were analyzed with an *S*(treatments × ages × sex) ANOVA identical to that used to assess plasma glucose on treatment day 10 (see above). The ages main effect was significant ($F_{[1,58]}$ = 4.13, $p < 0.05$), with plasma insulin levels higher in adults (60.32 ± 4.60 mg/dL) than in pups (49.70 ± 2.79 mg/dL). The treatments main effect was marginally significant ($F_{[1,58]}$ = 3.99, p = 0.051); the plasma insulin levels of pups and adults were marginally higher 4 h after an insulin injection (60.20 ± 4.26 mg/dL) than they were after a saline injection (49.82 ± 3.18 mg/dL).

Body weight on treatment days 1–10: pup injection versus adult injection

Differences in body weight were analyzed using an *S*(treatments × ages × sex) × treatment days ANOVA. There were two treatments (inject insulin or saline), two ages (treatment onset on postnatal day 9 or 60), two sexes, and 10 treatment days. Treatments, ages, and sex were between-group measures, and treatment days was a repeated measure.

The treatments main effect was not statistically significant, nor were there any significant interactions with treatment. Thus, for both pups and adults, the body weights of animals injected daily with 8 U insulin/kg did not differ from those of animals injected with saline.

The days × ages × sex interaction was significant ($F_{[9,522]}$ = 38.70, $p < 0.0001$) as was the linear component of this interaction ($F_{[1,58]}$ = 1861.38, $p < 0.0001$). As expected, the sex difference in body weight was larger for adults (males = 297.58 ± 2.53 g, females = 189.43 ± 1.54 g) than it was for pups (males = 33.03 ± 0.48 g, females = 32.12 ± 0.40 g), and the amount of weight gained across the 10-day period was greater for adult males (47 g) than it was for adult females (23 g).

Experiment 2 discussion

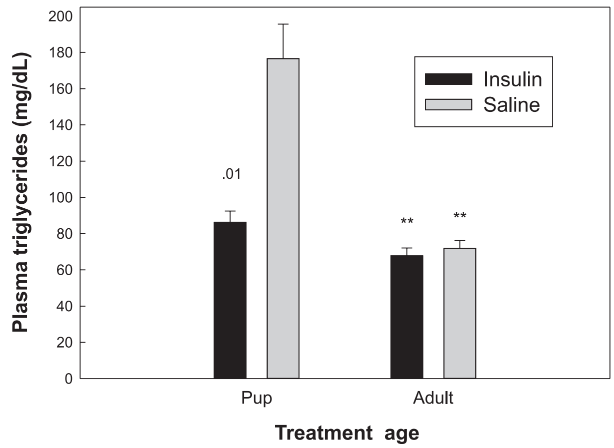
Daily injections of insulin in doses of 8 U/kg body weight caused no deaths to the pups (or adults) in the present study. These data replicate the findings of Experiment 1 as well as earlier findings in our laboratory (Thompson et al. 1997). In addition, no pups died while receiving insulin via a minipump. This is in sharp contrast with the findings of Experiment 1, where insulin delivered via a pellet killed 90% of the 9-day-old pups who received a 24-h dose of 5.7 U/kg and 30% of the pups who received a dose of 1.9 U/kg. The 8 U/kg minipump insulin dose that 9-day-old pups survived in Experiment 2 thus was 40% higher than the largest pellet dose in Experiment 1 and 321% higher than the smallest pellet dose.

The present data do not explain why pups tolerate insulin more readily from a minipump than from a pellet. The volume of fluid that remained in the minipumps at the end of the 10-day treatment indicated that the minipumps released the calculated dose of insulin. Regardless of what caused the difference in tolerance for insulin delivered via pellet versus minipump, however, the fact that all pups survived the minipumps indicates that the inability of preweaning pups to survive insulin-releasing pellets cannot be explained as an inability to tolerate continuous daily exposure to insulin.

The pups and adults consistently exhibited hypoglycemia 2 h after an insulin injection, and the degree of hypoglycemia did not diminish across the 10 treatment days. In contrast, pups who received insulin from a minipump exhibited hypoglycemia only on treatment day 2. On treatment days 5, 8, and 10, the glucose levels of pups carrying insulin minipumps could not be distinguished from control levels. Thus, pups given continuous insulin developed a compensatory mechanism that normalized their plasma glucose levels within 5 days of treatment initiation. This phenomenon is similar to that observed in pups who survived the insulin pellets in Experiment 1. The nature of the compensatory mechanism is not presently known, but some possibilities were suggested in the Experiment 1 discussion section.

Following the hypoglycemia that was evident 2 h after an insulin injection, both pups and adults exhibited an overcompensatory hyperglycemia at 4 h. Adults exhibited hyperglycemia on treatment day 2, the first day that glucose was measured, and they continued to be hyperglycemic during every subsequent 4-h glucose test. Pups took longer than adults to exhibit the hyperglycemia; although they never were hypoglycemic 4 h after an insulin injection, hyperglycemia did not appear until treatment day 5 in Experiment 2A and day 8 in Experiment 2B. Once an overcompensatory hyperglycemia appeared, however, it remained evident during every subsequent 4-h glucose test. Unlike the pups who received insulin injections, pups given insulin via a minipump never exhibited hyperglycemia. These animals were hypoglycemic on treatment day 2 and normoglycemic thereafter.

Fig. 6. Mean plasma triglycerides (±SE) on treatment day 10 for pups and adults injected with 0 or 8 U insulin/kg in Experiment 2B. Pups had higher triglyceride levels than adults. Injecting insulin lowered triglyceride levels 4 h later in pups but not in adults. **Lower than pups injected with insulin ($p < 0.01$).



Plasma triglyceride levels were measured as a second, insulin-sensitive metabolic indicator on treatment day 10. Similar to what occurred for plasma glucose levels, in both Experiments 2A and 2B, pups exhibited significant differences in plasma triglyceride levels depending on whether they received insulin via minipump or injection. For pups with minipumps, the plasma triglyceride levels of animals receiving insulin did not differ from those of saline control animals. In contrast, the plasma triglyceride levels of pups who received insulin by injection were significantly below those of control pups at 4 h postinjection. This observation suggests that pups receiving “chronic” insulin via a minipump become resistant to insulin’s normal effect of lowering plasma glucose and triglycerides. On the contrary, pups receiving “acute” insulin via a daily injection remain sensitive to the hormone and continue to respond with both hypoglycemia and hypotriglyceridemia.

Unlike the pups, adults injected with insulin did not exhibit hypotriglyceridemia. Their plasma triglycerides did not differ from control levels 4 h after the insulin injection on treatment day 10.

Pups exhibited higher baseline glucose levels than adults, a difference that was evident 2 and 4 h after a saline injection. Two hours after an injection of insulin, however, the glucose levels of pups fell significantly below those of adults. This hypoglycemia was evident even though pups feed primarily during the day, whereas adults are nocturnal feeders. Thus, the greater postinjection drop in plasma glucose occurred in pups despite their possibly having fed more recently than the adults. These data suggest that pups are less capable than adults of maintaining normoglycemia when confronted with an insulin challenge.

As in Experiment 1, there was little evidence that 10 days of insulin treatment affected body weight. In Experiment 2A, growth rates were identical for pups with insulin and saline minipumps, although pups in both minipump groups grew somewhat less rapidly than did pups who received injections. Pups injected with saline initially grew faster than did pups injected with insulin, but the weight difference disappeared by the ninth day of treatment. In Experiment 2B, insulin and saline injections had no differential effects on body weight for either pups or adults. The absence of any effect of chronic insulin on growth duplicates the results obtained in our earlier study with pups (Thompson et al. 1997). However, it differs from reports that hyperinsulinemia in pregnant rats and humans increases body weight in the offspring (Jones and Dayries 1990; Silverman et al. 1993; Buchanan and Kitzmiller 1994; Jones et al. 1995). It also differs from reports of increased body weight in adult rats following daily exposure to 9.8 U insulin/kg from a minipump for 7 weeks (Holmäng et al. 1995) or to daily injections of 40 U insulin/kg for 2 weeks (Roberts et al. 1994); however, we note that the insulin doses used in these investigations were higher than the 8 U/kg dose used in the present study and that insulin delivery continued for a longer time than the 10 days used here.

General discussion

In a previous study, we reported that giving rat pups insulin injections on postnatal days 9–20 lowered their plasma glucose for less than 6 h a day but that this exposure was nevertheless sufficient to attenuate the hypoglycemia produced by insulin injections given after the animals reached adulthood (Thompson et al. 1997). The physiological mechanisms responsible for this long-term effect remain unknown, although a down-regulation of insulin receptors is one possibility. Whatever the mechanisms, however, the present data suggest that the long-term consequences of exposing pups to chronic insulin via a minipump may be even more substantial than those produced by repeated injections. The fact that pups given insulin by minipump were normoglycemic by day 5, whereas pups injected with insulin continued to exhibit postinjection hypoglycemia after 10 days, suggests that insulin minipumps may induce a more substantial “insulin resistance” than do insulin injections.

One goal of the present studies was to find a safe way to continuously administer insulin to pups so that in the future, we may examine the effects of this early exposure on adult animals. The present studies indicate that subcutaneous insulin pellets are not suitable for this purpose, since rat pups do not tolerate them well. On the other hand, it will be feasible to compare the long-term effects of receiving equivalent doses of insulin via daily injections or an osmotic minipump. However, the two procedures will differ in a way other than the length of the daily exposure to insulin. Pups given daily injections experience a brief episode (<4 h) of hyperinsulinemia and hypoglycemia each day. In contrast, pups who receive insulin via a minipump rapidly reach a point where they are neither hyperinsulinemic nor hypoglycemic.

Investigations of the effects of insulin in rat pups may provide an animal model for the type 2 diabetes mellitus that often occurs in humans whose mothers develop gestational diabetes during pregnancy. In type 2 diabetes, circulating insulin levels typically are normal or even elevated (Weyer et al. 1999), but target tissues fail to respond to the hormone due to insulin resistance. Gestational diabetes, which complicates 4–6% of all pregnancies, resembles type 2 diabetes in that it is characterized by insulin resistance and hyperglycemia. Mothers with gestational diabetes expose the fetus to high levels of plasma glucose, which in turn stimulates increased insulin release from the fetal pancreas, i.e., chronic hyperinsulinemia. These children often become insulin resistant and are at high risk for developing type 2 diabetes (Silverman et al. 1998). It has been suggested that the fetal hyperinsulinemia experienced by these individuals is the cause of their subsequent metabolic derangements (Petit et al. 1993). The rat pups exposed to exogenous insulin in the present study appeared to become insulin resistant. Thus, the present model may complement other rat models of type 2 diabetes (Reed et al. 2000).

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